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Citation	Fems Microbiology Ecology, 2009, v. 69 n. 3, p. 449-460
Issued Date	2009
URL	http://hdl.handle.net/10722/65596
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1 **Response of bacterioplankton community structures to hydrological**
2 **conditions and anthropogenic pollution in contrasting subtropical**
3 **environments**

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16 **Key Words:** bacterioplankton, community structure, coastal environments,
17 hydrological conditions, anthropogenic pollution

18

19 **Running title:** Bacterioplankton community of contrasting coastal waters

20

1 **Abstract**

2 Bacterioplankton community structures under contrasting subtropical marine
3 environments (Hong Kong waters) were analyzed using 16S rRNA gene denaturing
4 gradient gel electrophoresis (DGGE) and subsequent sequencing of predominant
5 bands for samples collected bimonthly from 2004 to 2006 at five stations. Generally
6 bacterial abundance was significantly higher in summer than in winter. The general
7 seasonal variations of bacterial community structure as indicated by cluster analysis
8 of DGGE pattern were best correlated with temperature at most stations except for the
9 station close to a sewage discharge outfall, which was best explained by pollution
10 indicating parameters (e.g. biochemical oxygen demand). Anthropogenic pollutions
11 appear to have affected presence and intensity of DGGE bands at the stations
12 receiving discharge of primarily treated sewage. Relative abundance of major
13 bacterial species, calculated by relative intensity of DGGE bands after PCR
14 amplification, also indicated the effects of hydrological or seasonal variations and
15 sewage discharges. For the first time, a systematic molecular fingerprinting analysis
16 of bacterioplankton community composition was carried out along the environmental
17 and pollution gradient in subtropical marine environment and suggest that
18 hydrological conditions and anthropogenic pollutions altered total bacterial
19 community as well as dominant bacterial groups.

20

1 **Introduction**

2 In aquatic ecosystems, ubiquitous bacterioplankton is one of the major
3 components of food webs and play key roles in biogeochemical cycles and energy
4 flow. In the past two decades, various molecular techniques, such as denaturing
5 gradient gel electrophoresis (DGGE), terminal restriction fragment length
6 polymorphism, and automated ribosomal intergenic spacer analysis, have been used to
7 quantify microbial biodiversity. Previous evidence indicated that bacterioplankton
8 often show clear spatial patterns in terms of their distribution, abundance, and
9 phylogenetic diversity in marine ecosystems, which are affected by both
10 hydrodynamics and other anthropogenic factors. For example, Crump *et al.* (2004)
11 showed a strong influence of residence time on microbial biogeography along an
12 estuarine salinity gradient. Riemann and Middelboe (2002) showed pronounced
13 differences in bacterial community along a transect crossing the Skagerrak-Kattegat
14 front. General ecological theories, e.g. taxa-area relationships, were also verified in
15 microbial communities (Bell, *et al.*, 2005; Horner-Devine, *et al.*, 2004). It appears that
16 bacteria in aquatic ecosystems also have definable biogeography similar to that of
17 plants and animals (Martiny, *et al.*, 2006; Fuhrman, *et al.*, 2008).

18 In comparison to the work on spatial variations, there have been fewer studies on
19 temporal variations of marine bacterioplankton that cover the whole seasonal cycle in
20 coastal waters. For example, Pinhassi and Hagström (2000) examined the seasonal
21 distribution of marine bacterioplankton in the northern Baltic Sea using
22 whole-genome hybridization. Schauer *et al.* (2003) observed that the taxonomic

1 composition of the bacterioplankton in an oligotrophic coastal system of NW
2 Mediterranean Sea changed gradually throughout the year. Morris *et al.* (2005)
3 revealed temporal trends of bacterioplankton lineages in North Atlantic Ocean using
4 T-RFLP and quantitative rRNA hybridization. Kan *et al.* (2006) observed variable and
5 stable bacterial communities in the Chesapeake Bay in winter and summer,
6 respectively. Fuhrman *et al.* (2006) provided statistically robust demonstration of
7 temporal patterns of bacterioplankton in the coast of southern California and indicated
8 the biogeography of bacterioplankton might modulate function and response of
9 ecosystem. Notably, to our best knowledge, no seasonal study was performed in
10 subtropical coastal environments and/or with complex natural and anthropogenic
11 influence, e.g. sewage pollution.

12 Hong Kong (22° N, 113-114° E; Fig. 1), located at the southern coast of China,
13 has typical subtropical coastal environments with complex and seasonally varying
14 hydrography. A large amount of freshwater (annual flow of $308 \times 10^9 \text{ M}^3$) discharged
15 from the Pearl River to the western waters of Victoria Harbor, Hong Kong creates a
16 sharp environmental gradient across the harbor: salinity increases but nutrient loading
17 decreases from the west to the east (Yung, *et al.*, 1999). The eastern areas of Hong
18 Kong are predominantly affected by high salinity and nutrient-poor water from the
19 South China Sea. In addition, the middle part of Victoria Harbor, the water quality is
20 severely affected as a consequence of rapid population growth and economic
21 development, which introduced sewage discharge in the last several decades (Yung, *et*
22 *al.*, 1999). Hence, there are strong spatial and seasonal changes in the profiles of

1 nutrient, salinity, and other environmental factor in Hong Kong waters (Connell, *et al.*,
2 1998; Yin, 2003). These make Hong Kong waters to be a good system to study
3 microbial biogeography of subtropical coastal environments. In this study, we used
4 DGGE to study the changes in bacterioplankton community structure and relative
5 abundance of major bacterioplankton species in Hong Kong waters over a period of
6 two years (2004-2006). We aimed to illustrate the possible relationship between these
7 changes and various environmental parameters under contrasting environmental
8 conditions.

9

10 **Materials and Methods**

11 **Station characterization and sampling**

12 We selected 5 sampling sites in Hong Kong waters based on their environmental
13 characteristics, namely, Tung Lung Chau (TLC), Victoria Harbor East (VHE), Victoria
14 Harbor (VH), Victoria Harbor West (VHW), and Peng Chau (PC) (Fig. 1). According
15 to the results of a long-term monitoring by the Hong Kong Government
16 (<http://www.epd.gov.hk/>), TLC is a meso-trophic environment; PC is a nutrient-rich
17 estuarine environment; and VHE, VH and VHW are anthropogenically
18 nutrient-polluted stations. Detailed sampling station information (location, depth, etc.)
19 was shown previously (Zhang, *et al.*, 2007). At each sampling station, 6 liters (1 L
20 each for each replicate) of seawater from the surface (1 m below surface) and the
21 bottom (1 m above bottom) of the sea were collected bi-monthly from June 2004 to
22 April 2006. The samples were filtered through firstly a 1.0- μm -pore-size

1 polycarbonate membrane (47 mm diameter, Millipore) and subsequently a
2 0.22- μm -pore-size membrane (47 mm diameter, Millipore) to collect particle-attached
3 and free-living bacterioplankton, respectively. The membranes were immersed into
4 0.8 ml of extraction buffer (0.1 M of Tris-HCl, 0.1 M of $\text{Na}_2\text{-EDTA}$, 0.1 M of sodium
5 phosphate, 1.5 M of NaCl, 1% of CTAB) and stored on dry ice until DNA extraction.

6

7 **Determination of environmental parameters and bacterial abundance**

8 Temperature, salinity, pH, and dissolved oxygen content (DO) in the water column
9 were measured *in situ* using an YSI 6600 Sonde. The concentration of nutrients
10 including NH_4^+ , NO_2^- , NO_3^- , total phosphate (TP), silica (Si) was determined with a
11 Skalar San autoanalyzer for both the surface and the bottom water samples after
12 filtration through 0.7 μm GF/F (Whatman) filters (Knap, 1996). The concentration of
13 total nitrogen (TN) and dissolved nitrogen (DN) was measured with a Shimadzu TOC
14 analyzer, according to the protocols described by Knap et al. (1996). Suspended solid
15 content, turbidity, chlorophyll *a* (chl *a*) concentration, and biochemical oxygen
16 demand (BOD_5) were obtained from the Environmental Protection Department of
17 Hong Kong (<http://www.epd.gov.hk/>).

18 Fifty ml of each seawater sample were fixed with 4% of formaldehyde (final
19 concentration) and stored on dry ice for the quantification of bacterial abundance.
20 Bacterial abundances were determined using flow cytometry (COULTER EPICS XL,
21 Beckman) and SYBR Green I (Invitrogen) staining according to the methods
22 described by Gasol and del Giorgio (2000).

1

2 DNA extraction and PCR

3 Total DNA of particle-attached and free-living bacteria on the filters was extracted
4 and purified using proteinase K and sodium dodecyl sulfate concomitant with
5 chloroform extraction and isopropanol precipitation, following the protocol described
6 in details in Zhang *et al.* (2008). Bacterial 16S rRNA genes for DGGE were amplified
7 by a touch-down PCR program using the primer set 341F (5'-CCT ACG GGA GGC
8 AGC AG-3') and 907R (5'-CCG TCA ATT CMT TTG AGT TT-3') with a GC-clamp
9 attached to the forward primer ((Muyzer, *et al.*, 1993; Muyzer, *et al.*, 2004). The PCR
10 reaction mixtures (50 µl) contained 2 µl of template DNA, 1× *rTaq* buffer (TaKaRa),
11 0.2 µM of each primer, 100 µM of each deoxyribonucleoside triphosphate, 2.5 U of
12 *rTaq* DNA polymerase (TaKaRa). The amplification protocol included a denaturing
13 step at 95°C for 5 min, 10 touch-down cycles at 95°C for 30 sec, 65-55°C for 30 sec
14 (-1°C per cycle) and 72°C for 30 sec, 15 normal cycles at 95°C for 30 sec, 55°C for
15 30 sec and 72°C for 30 sec, and a final extension step of 72°C for 10 min.

16

17 DGGE and sequencing analysis

18 Similar to our previous study (Zhang, *et al.*, 2007), random checking indicated that
19 DGGE patterns of samples collected from surface and bottom seawater, as well as
20 samples from 6 replicates, were highly similar (Data not shown). Therefore, DGGE
21 analyses for large amount of samples, and subsequent statistical analyses based on
22 DGGE patterns, were performed using PCR products amplified from combined

1 environmental DNA from 6 replicates of surface and bottom samples at each station.
2 DGGE was carried out with a Bio-Rad Protean II system. PCR products were loaded
3 onto a 8% polyacrylamide gel with denaturing gradient of 45-75% (100% denaturant
4 = 7 M urea, 40% (vol/vol) formamide) and electrophoresis was performed at 125 V
5 for 18 h at 60°C in 1×TAE buffer. After electrophoresis, the gel was stained for 20
6 min using SYBR Gold (1:1,000 dilution; Invitrogen) and photographed with an Alpha
7 Imager 2000 (Alpha-Innotech-Corporation). The middle portion of each selected
8 DGGE band was excised, washed with Milli-Q water, and incubated in 50 µl of
9 Milli-Q water at room temperature for 4 h. Two µl of DNA from each excised band
10 were used as the template for the same PCR-DGGE analysis to check for the band
11 position and purity. PCR products were then purified and cloned into the vector with a
12 TOPO TA Cloning Kit (Invitrogen) according to the manufacturer's instructions. The
13 insertion of DNA fragments was confirmed by the same PCR-DGGE procedure. The
14 16S rRNA genes were sequenced from both ends using the primers M-13F and
15 M-13R with MegaBACE 500 (Amersham). The nucleotide sequences obtained with
16 the two primers were assembled using the Sequencher 4.2 (Gene Codes Corporation).

17 Phylogenetic affiliation of sequenced DGGE bands was determined by ARB
18 software (<http://www.arb-home.de/>; Ludwig, *et al.*, 2004). Sequences from DGGE gel,
19 as well as their close relatives determined with BLASTN program on NCBI
20 homepage (<http://www.ncbi.nlm.nih.gov/>), were input into ARB to update the
21 database (SLIVA Release 93). Sequence alignment was manually modified and the
22 neighbor-joining phylogenetic tree was constructed with bootstrapping of 1,000

1 replicates.

2

3 **Data analysis**

4 Previous studies suggest that major bands on DGGE gel represented dominant
5 bacterial species in *in situ* environments and band intensity was directly related with
6 the relative abundance of corresponding bacterial species within the sample (Murray,
7 *et al.*, 1996; Fromin, *et al.*, 2002). In the current study, we used identical experimental
8 protocols for all samples collected in two years, in which the biases introduced during
9 DNA extraction and PCR amplification were supposed to occur homogeneously. The
10 standardized protocols for all samples also made it possible to use band intensity as
11 relatively abundance of OTUs for calculation of diversity index and comparison
12 among samples (Fromin, *et al.*, 2002). DGGE band position and intensity were
13 determined using a GelCompar II software package (Applied Maths) and were
14 manually modified. Band matching was performed with 1.00% position tolerance and
15 1.00% optimization.

16 Cluster analysis for comparison of bacterial community structures was performed
17 based on the Pearson similarity correlation and the Ward dendrogramming method in
18 GelCompar II software package. The relationship between the measured
19 environmental parameters and the bacterial community structure revealed by DGGE
20 was studied using BIOENV analysis provided in PRIMER 5 software. BIOENV
21 analysis selects the environmental parameters that may best explain the community
22 pattern, maximizing the correlation between their respective similarity matrices with

1 application of a weighted Spearman's correlation coefficient.

2

3 **Nucleotide sequence accession numbers**

4 The 16S rRNA gene sequences obtained in this study were deposited in the GenBank
5 under the following accession numbers: EF655903-EF655910.

6

7 **Results**

8 **Environmental characterization**

9 As expected, Hong Kong waters showed clear seasonal patterns of temperature,
10 salinity, DO, and Chl *a* (Supplementary materials Fig. S1). Water temperature at all
11 five stations was usually higher in summer (26.5 ± 1.0 °C in Jun, Aug, and Oct) than
12 in winter (19.9 ± 1.7 °C in Dec, Feb, and Apr). Salinity decreased from the eastern
13 (34.1 ± 0.9 psu at TLC) to the western side (31.7 ± 2.5 psu at PC) and the variations
14 between summer and winter were clearer at PC than at TLC. DO concentration
15 increased from 5.5 ± 0.8 mg/L in summer to 7.5 ± 1.1 mg/L in winter. TLC always
16 showed lower concentrations of nutrients and BOD₅ than other four stations did
17 (Supplementary materials Fig. S1). Chl *a* was consistently low at TLC and higher in
18 some summer months at other four stations. Most environmental parameters (except
19 salinity, DO, suspended solids, and turbidity) of surface and bottom seawaters did not
20 differ largely and stratification was only observed in summer (Supplementary material
21 Fig. S1).

22 Bacterial abundances, determined by flow cytometry, varied from 0.18×10^6 to

1 2.56×10^6 in the surface seawater and from 0.16×10^6 to 2.34×10^6 in the bottom
2 seawater. Bacterial abundance also showed clear seasonal trends with higher
3 abundances from Apr to Oct and lower in Dec and Feb (Supplementary material Fig.
4 S2; One-way ANOVA, $p < 0.05$). However, the spatial difference of bacterial
5 abundance in Hong Kong waters was not clear in the sampling period.

6

7 **Seasonal pattern of bacterioplankton community**

8 The detected number of DGGE bands ranged from 7 to 27 in all samples investigated
9 (Fig. 2). Among 10 temporal patterns investigated [5 stations \times 2 populations
10 (particle-attached and free-living bacteria)], 7 of them showed the highest number of
11 DGGE bands in summer and the lowest in winter from 2004 to 2006. Both
12 particle-attached and free-living bacterial populations from TLC usually showed
13 higher band number in summer (e.g. Jun, Aug, and Oct of 2004 and 2005), resulting a
14 seasonal variation (One-way ANOVA, $p < 0.05$). However, only samples collected in
15 April always showed low band number in both particle-attached and free-living
16 bacteria. Nevertheless, no clear temporal trend was found for samples from the three
17 stations at Victoria Harbor (VHE, VH, and VHW) and PC. At the same time,
18 particle-attached and free-living samples did not always showed the same pattern at
19 the same station. For example, particle-attached bacteria at TLC in June 2005 showed
20 a relatively low apparent diversity (number of DGGE bands) while free-living
21 bacteria at the same sampling time showed a relatively higher diversity. The
22 inconsistency was observed for samples collected in February 2006 as well (Fig. 2).

1 Generally, bacterial community structures, revealed by cluster analysis of DGGE
2 pattern, showed clear seasonal patterns except for the western part of Victoria Harbor
3 (Fig. 3). Particle-attached (data not shown) and free-living bacterial (Fig. 3)
4 community structures at TLC, VHE, VH, and PC were grouped into two large clusters
5 mainly according to their sampling seasons. However, weak temporal trends were
6 observed for samples from VHW, where the samples from summer and winter
7 clustered together (Fig. 3). Bacterial community structure was more stable in summer
8 than in winter. Among 10 temporal dynamic patterns of bacterial community structure
9 investigated (5 stations \times 2 populations), samples collected in October and August
10 clustered together in 6 and 5 patterns, respectively. Only 2 patterns showed that the
11 samples collected at the same winter time (e.g. Dec, Feb or Apr) formed same cluster
12 (Fig. 3).

13 BIOENV analysis was used to correlate multivariate DGGE profiles with
14 environmental variables (Table 1). At each station, higher correlation values were
15 obtained for free-living bacteria than for particle-attached bacteria. Temperature
16 showed the highest correlation with bacterial community structure in 9 out of the 10
17 correlations (5 stations \times 2 bacterial populations). Furthermore, in two correlations
18 (particle-attached bacteria from VH and PC, Table 1), temperature was the only
19 parameter that best correlated with bacterial communities. However, at VHW, DO, TP,
20 and BOD₅ were observed as factors mostly correlated with bacterial community
21 structures, with correlation values of 0.727 and 0.802 for particle-attached and
22 free-living bacteria, respectively. Turbidity or suspended solid concentrations were

1 listed as significant environmental factors in 6 correlations. Nitrogen nutrient (NH_4^+ ,
2 NO_2^- , TN, and DN) was another contributing parameter affecting bacterial community
3 structures in Hong Kong waters (Table 1).

4

5 **Seasonal pattern of dominant bacterial species**

6 In total, eight major DGGE bands were sequenced, based on their intensity and
7 temporal variation, and their relatively abundance, compared to total PCR-amplified
8 bacterial 16S rRNA gene, was calculated (Fig. 4). They accounted for an average of
9 47% of the total band intensity of DGGE gels. Six of them were affiliated to
10 *Proteobacteria* with three belonged to the gamma subgroup, two to the alpha
11 subgroup and one to an uncultured delta subgroup. Other two DGGE bands were
12 affiliated in *Cyanobacteria* (*Synechococcus* sp.) and *Bacteroidetes* (*Cytophaga* sp.).
13 Relative density of six out of eight bands (except M-1 and M-2) showed significant
14 seasonal pattern (Fig. 5; One-way ANOVA, $p < 0.05$).

15 Sequence of the DGGE band M-1 showed 93% and 91% similarities to the 16S
16 rDNA sequence of an uncultured and a cultured *Legionella* sp., respectively.

17 Phylogenetic analysis based on the ARB database also indicated that it was closely
18 related with a group of *Legionella* spp. (Fig. 4). M-1 showed a lower occurrence in
19 particle-attached bacteria of three stations of Victoria Harbor than of other populations
20 and stations (One-way ANOVA, $p < 0.001$). The highest percentage of M-1 reached
21 20.3% of PCR-amplified 16S rRNA gene in the sample of free-living population at
22 VHW in October 2005 (Fig. 5). Band M-2 showed a high sequence identity to and

1 was clustered with uncultured *Roseobacter* spp. (Fig. 4). M-2 appeared as one of the
2 major groups with an average percentage of 15.3% of total amplicon in most of both
3 particle-attached and free-living bacterial communities in all the five stations (Fig. 5).
4 Sequences of M-3 and M-4, which shared 97.9% in sequence identity but were clearly
5 separated on DGGE gel, were grouped with *Glaciecola* spp. from cold environments
6 (Fig. 4). M-3 showed a higher abundance in winter seasons while M-4, in contrast,
7 showed higher percentages in summer seasons (One-way ANOVA, $p < 0.05$).
8 Furthermore, the highest relative abundance of M-3 and M-4 appeared at VHW
9 station. M-5 showed a high sequence identity and close phylogenetic relationship with
10 uncultured *Cytophaga* spp. and *Bacteroidetes* spp. from hypersaline ecosystem (Fig.
11 4). The detectable signals of M-5 came from the samples collected at PC and VH
12 (only free-living bacteria) in winter (Fig. 5). M-6 showed a 100% sequence identity to
13 an uncultured *Synechococcus* sp. (AB294981) and was clearly grouped within a group
14 of cultured *Synechococcus* spp.. Significantly, it bloomed in summer, especially for
15 the particle-attached bacterial populations, with the highest percentage (30%)
16 recorded at TLC in August 2004 (Fig. 5; One-way ANOVA, $p < 0.05$). For free-living
17 bacteria, no signal of M-6 was detected for samples from three stations at Victoria
18 Harbor (Fig. 5). M-7 did not have any cultivated relatives with BLASTN search in the
19 Genbank and showed 99% sequence identity with a clone from HOT station, USA. It
20 was abundant in summer season (One-way ANOVA, $p < 0.001$) and showed the highest
21 percentages in particle-attached fractions of VHW (Fig. 5). Despite the low sequence
22 identity (91%), M-8 was related to *Azospirillum* spp. based on the phylogenetic

1 analysis (Fig. 4) and had higher abundances in summer at VHW (Fig. 5).

2

3 **Discussion**

4 **Effects of hydrological conditions on bacterial communities in Hong Kong waters**

5 The combined effects of annual river discharge, rainfall, and monsoon winds
6 determined the seasonal profiles of temperature, salinity, and other environmental
7 parameters in Hong Kong waters during our sampling period (Supplementary material
8 Fig. S1), which was also recorded in previous studies (Yin, 2002; Yin, 2003). Overall,
9 about 80% of the annual discharge from Pearl River and rainfall occurred in summer
10 seasons with the maxima in June, July, and August (Yin, 2003). Although easterly
11 winds occur throughout the year, northeasterly to easterly winds blow in winter and
12 southerly to southwesterly winds in summer. As a result, during the period of
13 December to April, the China Coastal Current that originates from the north
14 dominates the Hong Kong coastal water circulation. In summer, the southwest
15 monsoon drives upwelling along the coast, together with the Pearl River discharge
16 and maximal rainfall (Yin, 2003). Furthermore, data of long-term observation from
17 the Environmental Protection Department of HKSAR and other studies indicated
18 three contrasting environments, meso-trophic coast (TLC), anthropogenic polluted
19 coast (VHE, VH, VHW), and eutrophic estuary (PC), were developed from the east to
20 the west along Victoria Harbor in Hong Kong waters, . Our previous study showed
21 clear spatial variations of particle-attached and free-living bacterial communities
22 using DNA fingerprinting and clone library analyses (Zhang, *et al.*, 2007). The

1 present study indicated the bacterioplankton in Hong Kong waters showed clear
2 seasonal patterns as well.

3 Generally, the effects of hydrological conditions on bacterial populations were
4 observed in Hong Kong waters. Firstly, bacterial abundances differed between
5 summer and winter seasons, which are mainly due to the influence of annual variation
6 of temperature (Supplementary material Fig. S1). Clear seasonal patterns of
7 community structures of particle-attached and free-living bacterial populations were
8 observed at TLC, VHE, VH, and PC using cluster analysis of DGGE gel (Fig. 3). This
9 indicated substantially different bacterial populations existed in different seasons. Our
10 finding at subtropical Hong Kong waters was consistent with those of global marine
11 environments with totally different hydrological conditions, e.g. the Blanes Bay
12 (Temperate Mediterranean Sea; Alonso-Sáez, *et al.*, 2007; Schauer, *et al.*, 2003,
13 Pinhassi, *et al.*, 2006), the Gulf of Trieste (Temperate Adriatic Sea; Celussi &
14 Cataletto, 2007); the Chesapeake Bay (Subtropical-temperate Atlantic; Kan, *et al.*,
15 2006; Crump, *et al.*, 2007; Kan, *et al.*, 2007), the San Pedro Harbor (Subtropical
16 Pacific; Fuhrman, *et al.*, 2006), the Banyuls-sur-mer Bay (Temperate Mediterranean
17 Sea; Ghiglione, *et al.*, 2005), the English Channel (Mary, *et al.*, 2006), the Bermuda
18 Sea (Morris, *et al.*, 2005), the Baltic Sea (Pinhassi & Hagstroem, 2000; Riemann, *et*
19 *al.*, 2008), the North Sea (Sapp, *et al.*, 2007). Most of the studies related seasonal
20 bacterial community dynamics with environmental parameters (e.g. temperature,
21 salinity, etc.). Indeed, in our study, BIOENV analysis showed that temperature was
22 one of driving forces for the variations detected by DGGE (Table 1). However, some

1 investigations based on various lake systems showed less or no seasonal pattern of
2 planktonic bacterial composition (Lindstroem, 1998; Yannarell, *et al.*, 2003; Kent, *et*
3 *al.*, 2004; Yannarell & Triplett, 2005). One possible reason of this contrasting
4 phenomena may be the closed versus open nature of the systems.

5 Furthermore, only samples at TLC showed a clear seasonal pattern of DGGE
6 band number (apparent diversity) of bacterial populations (Fig. 2). Due to the fact that
7 TLC is the cleanest station, we supposed that the clear seasonal pattern of bacterial
8 apparent diversity at TLC came from its pollution conditions and calculation of these
9 ecological parameters from DGGE. The calculation of apparent diversity simply
10 depends on the total number of DGGE bands, which include the weak bands (minor
11 bacterial groups) as well. Bacterial community structure analysis, which was based on
12 the cluster analysis of similarity matrix from DGGE gel pattern, considered the band
13 intensity on DGGE gel (e.g. abundant bacterial groups might show high band
14 intensity; Muyzer & Smalla, 1998; Fromin, *et al.*, 2002). This indicated that the
15 communities of major bacterial groups at TLC, VH, VHE, and PC followed a general
16 seasonal pattern, while other factors (e.g. pollutions, see discussion below)
17 “stimulated or repressed” minor bacterial groups, changing the species richness but
18 not disturbing the seasonal pattern of general bacterial community structures (Fig. 3).
19 Meanwhile, we cannot exclude the facts that bacterial diversity displayed on DGGE
20 gel was not representative of all bacterial community due to limitation of DGGE gel
21 resolution.

22

1 **Effects of anthropogenic pollutions on bacterial communities in Hong Kong**
2 **waters**

3 Since 1970s, the Hong Kong waters, especially in Victoria Harbor area, have been
4 severely polluted by domestic sewage and industrial effluents. In 1997, the estimated
5 loading of total BOD, total suspended solids, and total toxic metals into the Harbor
6 area was about 340 tons, 280 tons, and 3000 kg per day, respectively (Yung, *et al.*,
7 1999). There are 12 outfalls from 11 sewage screening plants and one Stonecutters
8 Island Sewage Treatment Works which discharge about 1.7 million M³ primarily
9 treated wastewater into the Harbor (near VHW, Fig. 1). A previous study on spatial
10 diversity of bacterioplankton in the Hong Kong waters strongly indicated the
11 influences of anthropogenic pollutions (Zhang, *et al.*, 2007). For example, the
12 sequences of fecal indicators of *Bacteroides* and *Arcobacter* were only observed in the
13 clone libraries from VH, but not from TLC and PC. Temporal patterns of bacterial
14 communities, revealed in the present study, showed possible effects of pollution as
15 well. Bacterial community structure at VHW, the closest station to one of the largest
16 sewage treatment works, was the only one that did not show clear seasonal patterns in
17 bacterial community structures among the five stations. Samples, especially of
18 free-living bacteria, from summer and winter mixed together in the cluster analysis
19 (Fig. 3). Meanwhile, BIOENV analysis showed VHW was the only station in which
20 bacterial community could be highly correlated with DO and BOD₅ (Table 1). This
21 suggested that the consistent and routine discharge of preliminarily treated sewage
22 near VHW substantially affected the bacterial community and disturbed the natural

1 patterns of bacterial community structure produced by seasonal changes of the
2 ecosystem. Furthermore, in the Hong Kong waters, a relatively small scale area,
3 apparent diversity (the number of detectable bands on DGGE gel) showed clear
4 spatial variations for all sampling times (Fig. 2). A simple explanation of the variation
5 was the influence of a consistent and large amount of pollution discharge in Victoria
6 Harbor area. TLC was the least affected by the pollution discharge and might be a
7 reference site in comparison to other stations that have been receiving pollution
8 discharge routinely. The nutrients, along with sewage discharge, might stimulate or
9 repress bacterial growth and, consequently, affected specific groups of bacteria, which
10 appeared as presence or absence of weak bands on DGGE gel. Although only eight
11 major bands were excised and sequenced in the present study, a detailed previous
12 study (Zhang, *et al.*, 2007) in which 28 bands (including weak bands) were sequenced
13 supported the explanation.

14 To our best knowledge, the present study, for the first time, documented the
15 long-term effects (disturbing seasonal pattern of bacterial community structure) of
16 pollutions to marine bacterioplankton. Furthermore, our study indicated that BOD₅
17 (combined with other nutrient parameters) maybe an appropriate indicator when
18 considering the anthropogenic effects on microbial biogeography.

19

20 **Dominant bacterial groups in subtropical Hong Kong waters**

21 Previous studies showed that *Roseobacter* spp. and its close relatives were one of the
22 major marine bacterial lineages in coastal areas and played very important roles in

1 global carbon and sulfur cycle and climate (Selje, *et al.*, 2004; Buchan, *et al.*, 2005).
2 Our results indicated that *Roseobacter* spp. was also abundant in the subtropical
3 coastal Hong Kong area, with a high abundance of M-2 constituting 15.3% (range
4 4.8-27.9%) of the total band intensity in the DGGE profiles (Fig. 5). Furthermore, the
5 present study showed *Roseobacter* sp. was rather consistently distributed within two
6 summer-winter cycles with a clear temperature variation, which was differed from
7 previous studies (Buchan, *et al.*, 2005; Kan, *et al.*, 2007). Therefore, our study
8 suggested that *Roseobacter* might play more important roles than what we previously
9 thought in global carbon and sulfur cycle because they might be more widely
10 distributed and less sensitive to temperature changes. However, we (this study and
11 Zhang, *et al.*, 2007) did not recover the other important marine bacterial group SAR
12 11 in Hong Kong waters, although it was observed frequently at coastal area
13 (Pommier, *et al.*, 2005).

14 Clear spatial and temporal patterns were observed for another abundant coastal
15 species, *Synechococcus* sp. (M-6) (Fig. 5). The high percentages of *Synechococcus* in
16 particle-attached population (>1.0 μm) and at TLC were in good agreement with their
17 cell size and aggregation in *in situ* environments and the facts that TLC is the most
18 oceanic environments with the least effects of fresh water discharge (Fig. 1).
19 Furthermore, seasonal patterns showed they always appeared in summer in Hong
20 Kong waters (Fig. 5). Previous studies on spatial diversity of total bacteria and
21 temporal dynamics of cyanobacteria using clone library analysis verified the
22 conclusion from DGGE pattern (Zhang, *et al.*, 2007). A recent multiyear investigation

1 in the Chesapeake Bay revealed similar temporal distribution pattern of
2 *Synechococcus*-type of cyanobacteria (Kan, *et al.*, 2007).

3 On DGGE gel, two major bands (M-3 and M-4) were clearly separated and
4 showed different intensity in each samples, although their sequences had 98% of
5 similarity and both were closely related to *Glaciecola* sp. The different temporal
6 patterns of M-3 and M-4 excluded the possibility that they were from the same
7 bacterial strain. The two *Glaciecola* spp. averagely accounted for 21% of all bacterial
8 signals on DGGE gel and in some samples (e.g. free-living bacteria at VHW), the
9 percentages were higher than 40%. Phylogenetic analysis indicated that our
10 *Glaciecola* spp. were similar to those isolated from cold environments. Previously,
11 strains or environmental clones belonging to *Glaciecola* sp. were usually isolated
12 from polar or sub-polar seas (Bowman, *et al.*, 1998; Brown & Bowman, 2001; Van
13 Trappen, *et al.*, 2004). The only exception was that Alonso-Sáez *et al.* (2007) found
14 the blooming of *Glaciecola* from north-west Mediterranean coastal waters sampled in
15 July, 2003. This suggested that some *Glaciecola* spp. might survive, adapt and bloom
16 in much warmer waters than previously thought. Our study also indicated that
17 bacterial microdiversity might be a possible reason for the adaption of *Glaciecola* spp.
18 for less than 2 % sequence difference of their 16S rRNA genes. Similar to previous
19 study of *Prochlorococcus*, the diversification of different “ecotypes” in the same
20 “species” of *Glaciecola* might help them confounding viral attack and protistan
21 grazing (Rocap, *et al.*, 2003).

22 The possible pollution-related bacteria were detected and showed clear spatial

1 and temporal dynamics. *Cytophaga* sp. (M-5) was supposed to be an important
2 utilizer of organic matters in the ocean (in the Hong Kong waters, mainly originated
3 from sewage and river discharge), and was critical in carbon budgets and cycles
4 (Kirchman, 2002). Although several studies found that certain *Cytophaga* spp. showed
5 seasonal patterns with maximum abundance in winter, very few studies investigated
6 their seasonal distribution in marine ecosystems (Riemann & Middelboe, 2002). Our
7 results indicated that *Cytophaga* sp. was abundant only at PC and VH in winter season
8 (Fig. 5), which was consistent with the observations in fresh water systems (Riemann
9 & Middelboe, 2002). The other major bacterial group in Hong Kong waters was M-1.
10 Although relatively low similarity among M-1 and known *Legionella* sequences in
11 public database, our study was similar to previous studies (Atlas, 1999) of *Legionella*
12 spp. that M-1 was more abundant at VHW and VH, which are close to the sewage
13 outfall. The extremely high percentages (about 30%) of *Legionella*-like bacteria in
14 certain areas (e.g. VHW) at certain times (e.g. Oct, 2005) should be further
15 investigated and evaluated carefully.

16

17 **Conclusion**

18 Being one of the few long-term spatio-temporal studies on marine bacterioplankton,
19 the present study showed variations of particle-attached and free-living bacterial
20 community at different sites with contrasting environments in a subtropical coastal
21 area. Possible combined effects of hydrological conditions and anthropogenic
22 pollutions on bacterial community were observed: hydrological effects determined the

1 general bacterial community structure while anthropogenic pollutions affected nearby
2 bacterioplankton in Hong Kong waters. Dominant bacterial species, determined by
3 sequencing major DGGE bands and clone library (Zhang, *et al.*, 2007), in Hong Kong
4 waters were *Proteobacteria*, *Cyanobacteria* and *Bacteroidetes*. Temporal variation of
5 eight dominant bacterial species indicated a controlling mechanism of natural and/or
6 anthropogenic influence in coastal area.

7

1 **Acknowledgments**

2 We would like to thank the Environmental Protection Department, Hong Kong
3 Government for providing environmental monitoring data and critical comments from
4 Prof. Paul Harrison and Dr. Hongbin Liu. Technical assistances of Tam Yin Ki, On On
5 Lee, Ying Xu, Xiangcheng Yuan, Dongmei Li, and Bingzhang Chen were grateful
6 appreciated. This study was funded by HKSAR governmental grant (AoE04/04-02) to
7 P-Y Qian.

8

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9

1 Table 1. BIOENV analysis showed the correlations (Corr.) between bacterial
 2 community structure and environmental (Env.) factors. T: temperature, Sal:
 3 salinity, Turb: turbidity, SS: suspended solid, Chl *a*: chlorophyll *a*, DO: dissolved
 4 oxygen, BOD₅: 5-day biochemical oxygen demand, DN: dissolved nitrogen, TN:
 5 total nitrogen, TP: total phosphate.

6

	Particle-attached bacteria		Free-living bacteria	
	Corr.	Env. factors	Corr.	Env. factors
TLC	0.551	T, Sal, NH ₄ ⁺ , NO ₂ ⁻ , Turb	0.705	T, NH ₄ ⁺ , Turb
	0.493	T, NH ₄ ⁺ , Turb	0.693	T, NH ₄ ⁺ , Si, Turb
	0.491	T, NH ₄ ⁺ , NO ₂ ⁻ , Turb	0.670	NH ₄ ⁺ , Turb
VHE	0.701	T, SS	0.755	T, NO ₂ ⁻ , DN, SS
	0.643	T, Si, SS	0.754	T, NO ₂ ⁻ , Si, TN, SS
	0.630	T, NO ₂ ⁻ , DN, SS	0.751	T, Sal, NO ₂ ⁻ , DN, SS
VH	0.500	T	0.706	T, Sal, NO ₂ ⁻ , SS
	0.467	T, Sal	0.703	T, NO ₂ ⁻ , TP, SS, Chl <i>a</i>
	0.449	T, Sal, NO ₂ ⁻	0.687	T, Sal, pH, NO ₂ ⁻ , SS
VHW	0.727	T, DO, NH ₄ ⁺ , TP, Chl <i>a</i>	0.802	DO, NH ₄ ⁺ , TP, Turb, BOD ₅
	0.727	T, TP	0.787	DO, NH ₄ ⁺ , NO ₂ ⁻ , TP, BOD ₅
	0.727	T, DO, TP	0.779	T, NH ₄ ⁺ , NO ₂ ⁻ , TP, BOD ₅
PC	0.512	T	0.528	T, NO ₂ ⁻
	0.506	T, Si	0.502	T, Sal, NO ₂ ⁻
	0.467	T, Sal	0.484	T, Sal, NO ₂ ⁻ , Si

1 Fig. 1. Map showing five sampling stations in Hong Kong waters. The large sewage
2 treatment works is indicated with ★ (near VHW). TLC: Tung Lung Chau;
3 VHE: Victoria Harbor East; VH: Victoria Harbor; VHW: Victoria Harbor West;
4 PC: Peng Chau.

5 Fig. 2. Temporal variations of particle-attached bacterial apparent diversity (number
6 of DGGE bands) at Tung Lung Chau (TLC) and Victoria Harbor (VH) of
7 Hong Kong waters.

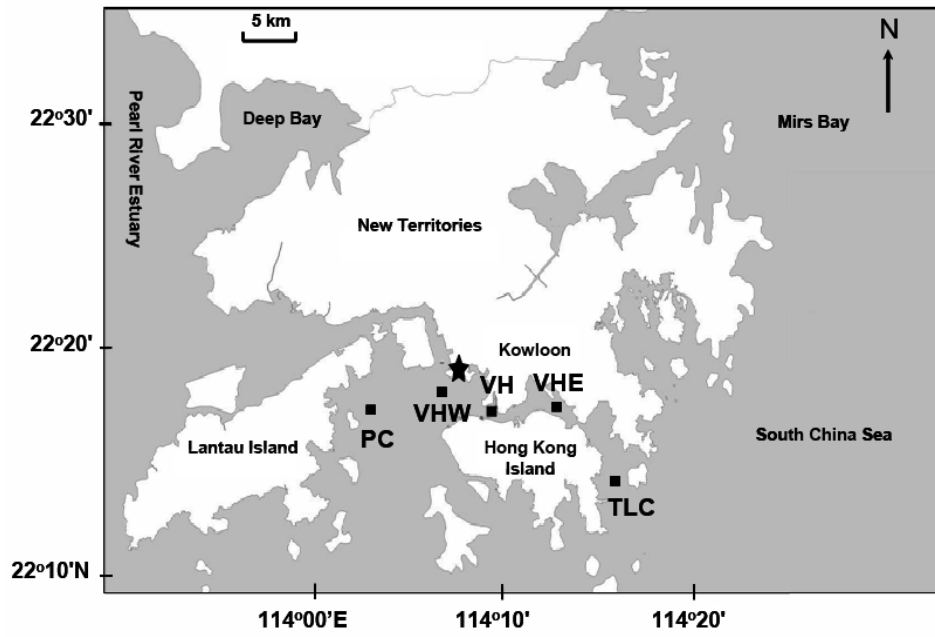
8 Fig. 3. DGGE patterns and cluster analyses of free-living bacterial community
9 structure at Tung Lung Chau (TLC) and Victoria Harbor West (VHW) stations
10 in Hong Kong waters from 2004 to 2006. Samples collected in summer (Jun,
11 Aug, and Oct) and winter (Dec, Feb, and Apr) were indicated.

12 Fig. 4. Neighbour-joining phylogenetic tree for bacterial 16S rRNA gene sequences
13 retrieved from DGGE. Bootstrap values (other than 100% were indicated at
14 nodes) were based on an analysis of 1,000 re-sampling using ARB software.
15 The scale bar represents 10% nucleotide sequence difference. *Bacillus*
16 *naganoensis* (Firmicutes) was used as an out-group.

17 Fig. 5. Temporal patterns of relative abundance of sequenced DGGE bands at five
18 stations in Hong Kong waters. Relative abundance was indicated with the
19 percentage of intensity of each DGGE band to the intensity of all DGGE bands
20 of each sample. The possible phylogenetic affiliations for sequences from
21 DGGE gel were indicated. Refer to Fig. 1 for site abbreviations.

22

1 Fig. 1.

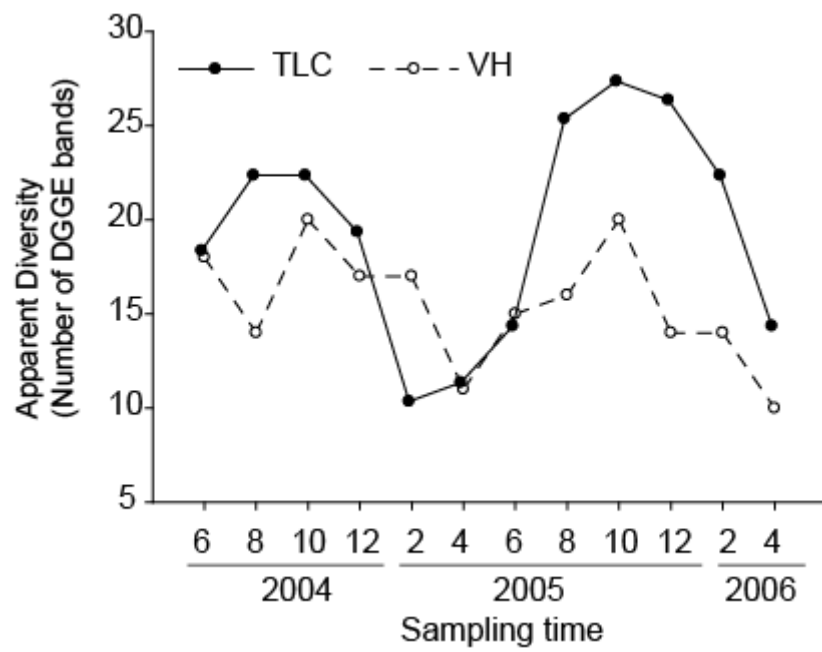


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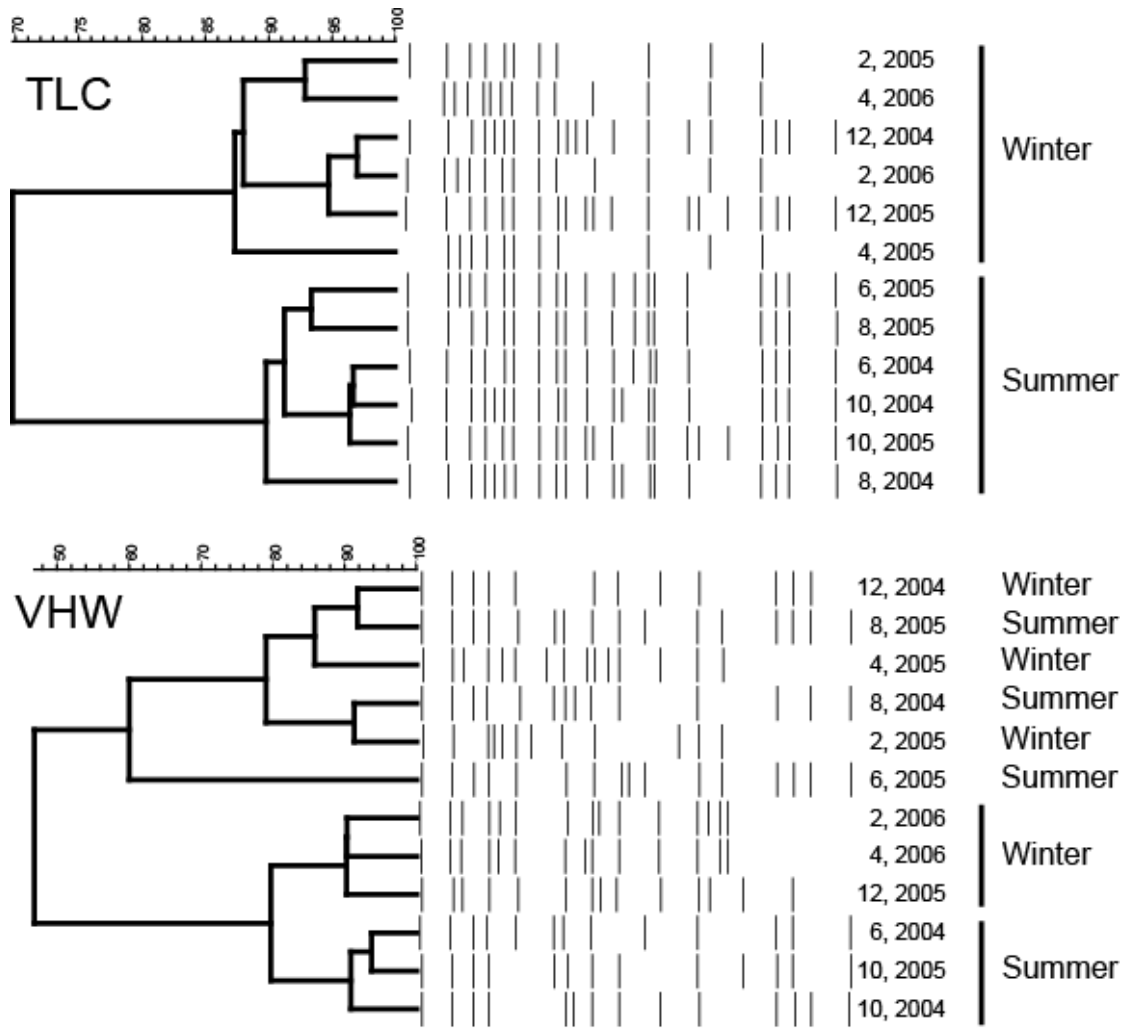
1 Fig. 2.



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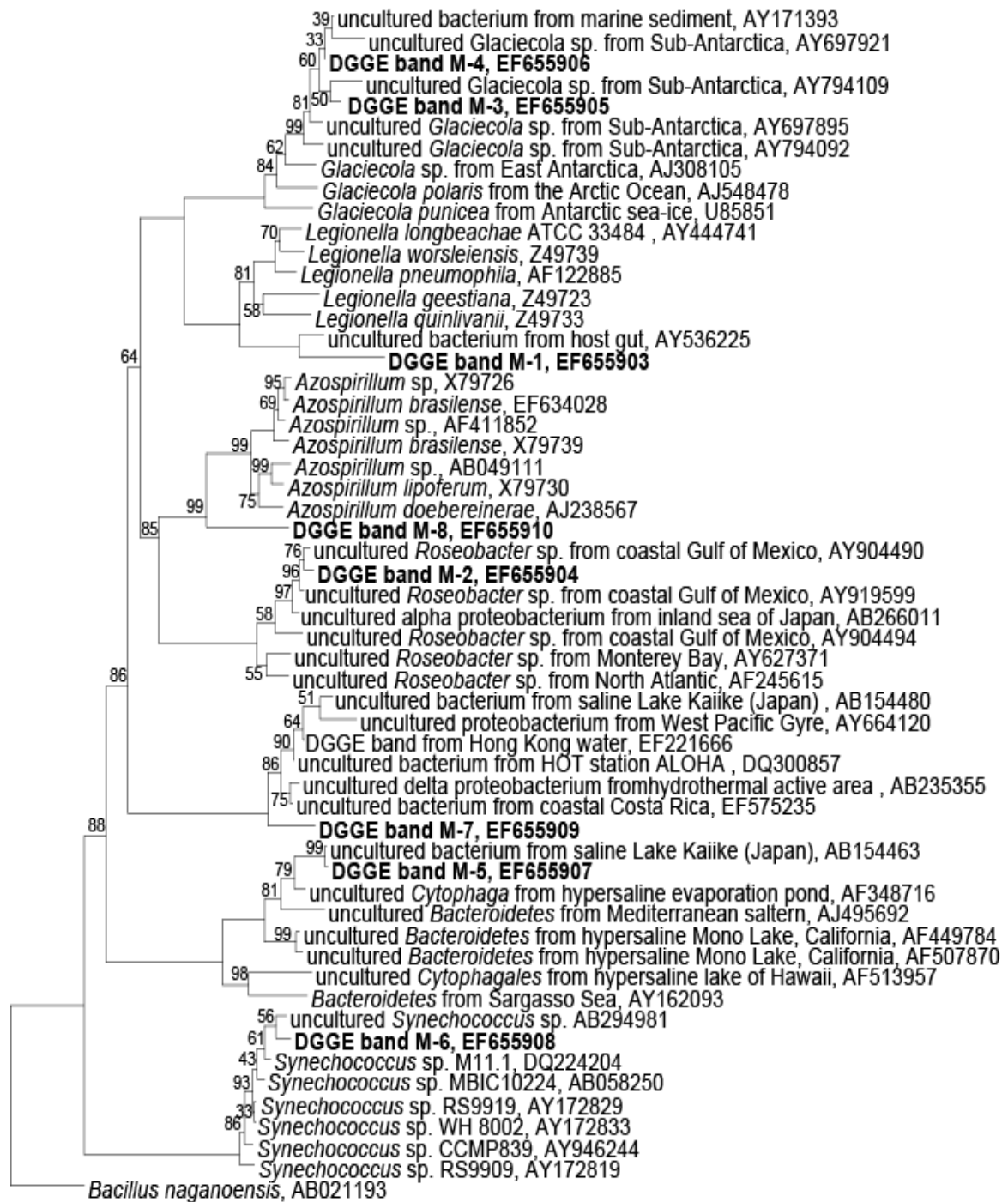
1 Fig. 3.



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3

1 Fig. 4.

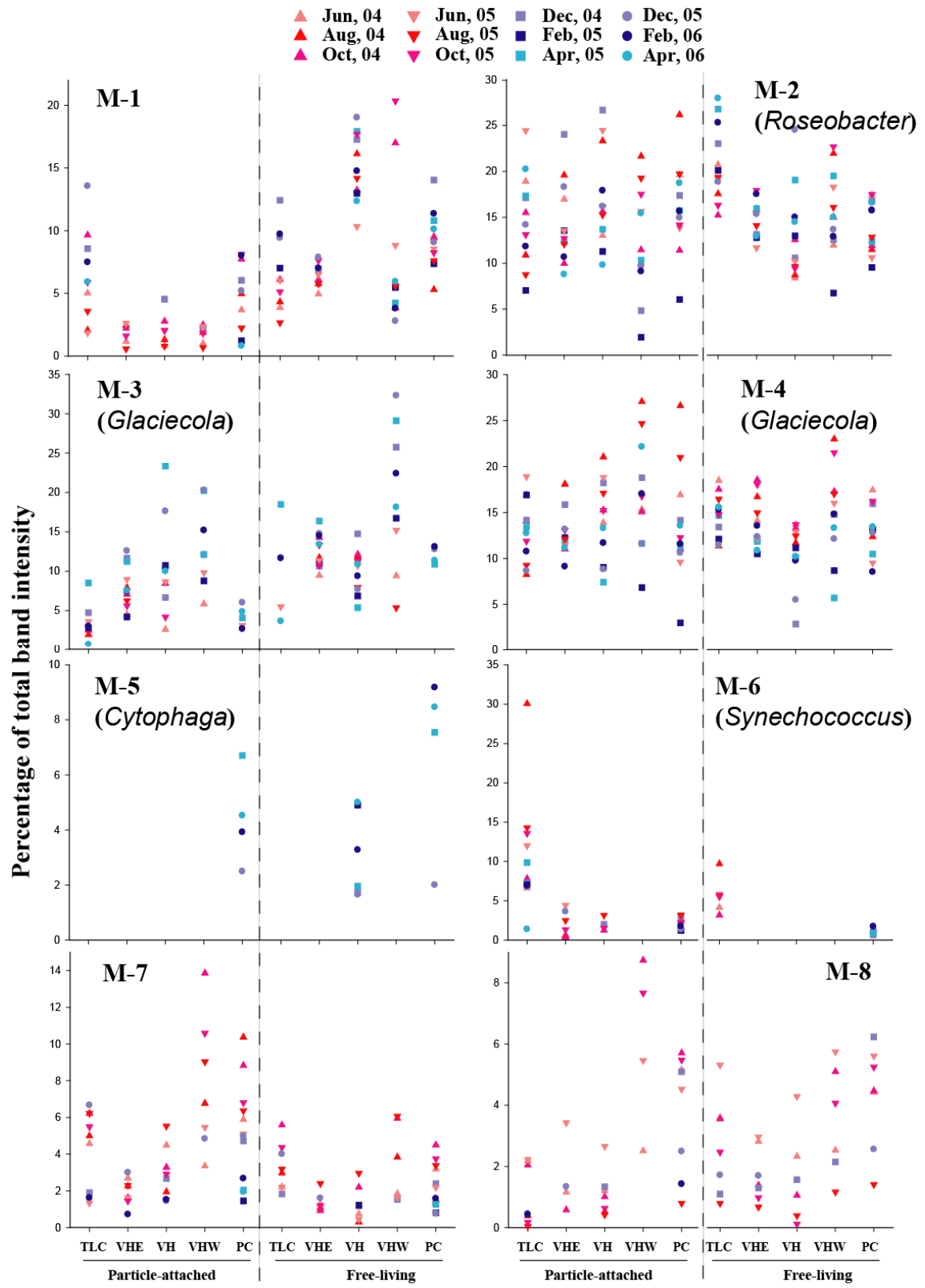


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0.10

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1 Fig. 5



2

3